# CD70 Is Selectively Expressed on Th1 but Not on Th2 Cells and Is Required for Th1-Type Immune Responses

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The interaction between CD27 and CD70 provides a costimulatory signal for T-cell survival. Although the role of CD27 signaling in $CD8^+$ T cells has been well defined, its role in $CD4^+$ T cells is relatively unknown. Here, we report that CD70 is specifically expressed on differentiated T-helper (Th)1 cells, but not on Th2 cells. Upon activation, CD70 expression increased markedly on Th1 cells, but remained undetectable on Th2 cells. We demonstrate that CD27 is involved in naive T-cell expansion in Th1-type, but not in Th2-type, immune responses as *in vivo* treatment with anti-CD70 monoclonal antibody at induction resulted in a significant reduction of delayed-type and contact hypersensitivity responses, but not asthmatic responses. In both Th1-type responses, during the priming phase, CD70 was detected at earlier time points on dendritic cells (DCs) and at later time points on $CD4^+$ T cells. Our results indicate that CD70 may be useful as a marker to distinguish Th1 from Th2 cells. More importantly, CD27 function may be controlled by the differentially regulated kinetics of CD70 expression on DCs and $CD4^+$ T cells, and Th1 cell-specific CD70 expression may be involved in an amplification loop for polarized Th1-type immune responses through T cell–T cell interactions.

*Journal of Investigative Dermatology* (2011) **131,** 1252–1261; doi:10.1038/jid.2011.36; published online 14 April 2011

## INTRODUCTION

CD27 is expressed on thymocytes, as well as on naive $CD4^+$ and $CD8^+$ T cells (Borst *et al.*, 2005; Watts, 2005; Nolte *et al.*, 2009). Its unique ligand CD70 is transiently expressed on activated T cells, B cells, and dendritic cells (DCs), and CD27 function is controlled by a tightly regulated CD70 expression *in vivo* (Oshima *et al.*, 1998; Tesselaar *et al.*, 2003; Borst *et al.*, 2005; Bullock and Yagita, 2005; Watts, 2005; Sanchez *et al.*, 2007; Nolte *et al.*, 2009). CD27 provides a costimulatory signal for T cells by supporting the survival of activated T cells rather than by affecting cell division (Hendriks *et al.*, 2000, 2003; Borst *et al.*, 2005; Peperzak *et al.*, 2010). A number of studies have examined the costimulatory effect of CD27/CD70 signaling on $CD8^+$ T cells *in vivo* using viral infection, cardiac allograft, tumors rejection, and major histocompatibility complex class I-restricted delayed-type hypersensitivity (DTH) models, in which CD27 has a critical role for $CD8^+$ T cells in the accumulation of cytolytic effector cells and in memory formation (Borst *et al.*, 2005; Watts, 2005; Nolte *et al.*, 2009).

$CD4^+$ T cells can be divided into T-helper type 1 (Th1) and type 2 (Th2) cells according to distinct profiles of cytokine production (Abbas *et al.*, 1996; Ho and Glimcher, 2002). Th1 cells produce IFN-γ, whereas Th2 cells produce IL-4, IL-5, and IL-13. Several surface molecules have been shown to be differentially expressed between Th-cell subsets. For example, the IL-12 receptor β2 chain, IL-18R, chemokine receptors CXCR3 and CCR5, Tim-3, and natural killer group 2 molecules are preferentially or selectively expressed on Th1 cells, whereas the T1/ST2, CCR3, CCR4, and inducible T-cell co-stimulator molecules are mainly found on Th2 cells (Szabo *et al.*, 1997; D'Ambrosio *et al.*, 1998; Lohning *et al.*, 1998; Sallusto *et al.*, 1998; Xu *et al.*, 1998; McAdam *et al.*, 2000; Meyers *et al.*, 2002; Monney *et al.*, 2002).

The role of CD27/CD70 interactions in Th1 and Th2 cell-mediated immune responses is unclear. CD27−/− mice infected with the influenza virus showed impaired accumulation of both $CD4^+$ T and $CD8^+$ T cells in the lung and spleen, respectively (Hendriks *et al.*, 2000). However, no difference in the percentages of IFN-γ-producing $CD4^+$ T cells was observed between CD27−/− and CD27+/+ mice. Nevertheless, CD27−/− mice immunized with the intranasal ovalbumin (OVA) protein showed decreased frequency of IFN-γ- and IL-2-producing $CD4^+$ T cells (Xiao *et al.*, 2008). Similarly, B cell-specific CD70 transgenic mice showed marked increases in the number of $CD4^+$ T cells that produced more IFN-γ but similar levels of IL-2 and

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*Abbreviations: CFSE, carboxyfluorescein diacetate succinimidyl ester; CHS, contact hypersensitivity; DC, dendritic cell; DLN, draining lymph node; DNBS, 2,4-dinitrobenzene-sulfonic acid; DTH, delayed-type hypersensitivity; OVA, ovalbumin; PMA, phorbol 12-myristate 13-acetate*

*Received 27 July 2010; revised 8 December 2010; accepted 19 January 2011; published online 14 April 2011*

tumor necrosis factor-α compared with wild-type mice (Arens *et al.*, 2001). In lymphocytic choriomeningitis virus infection, CD27 signaling in $CD4^+$ T cells enhanced the secretion of IFN-γ and tumor necrosis factor-α, leading to destruction of the splenic architecture (Matter *et al.*, 2006). Intriguingly, anti-CD70 mAb treatment significantly reduced the proinflammatory cytokines IL-6, tumor necrosis factor-α, and IFN-γ in an experimental inflammatory bowel disease model, whereas it did not affect tumor necrosis factor-α, IFN-γ, IL-4, IL-5, or IL-10 production in an experimental allergic conjunctivitis model (Sumi *et al.*, 2008; Manocha *et al.*, 2009). Thus, it is still unclear whether CD27/CD70 signaling is involved in $CD4^+$ T-cell priming, differentiation, or ''polarization'' *in vivo*.

In this study, we investigated the expression of CD70 on Th1 and Th2 cells derived from OVA-specific T-cell receptor transgenic mice, and the contribution of CD70 to OVA-specific Th1- and Th2-type immune responses *in vitro* and *in vivo*. We further investigated the role of CD27/CD70 signaling in contact hypersensitivity (CHS) by examining the effect of neutralizing anti-CD70 mAb *in vivo*.

## RESULTS

### Expression of CD27 and CD70 on Th1 and Th2 cells

We first examined the expression of CD27 and CD70 on activated Th1 and Th2 cells. Upon stimulation with ionomycin plus PMA (phorbol 12-myristate 13-acetate), Th1 and Th2 cell lines produced IFN-γ and IL-4, respectively (Figure 1a). CD27 was expressed on almost all resting Th1 and Th2 cells, but was partly downregulated by stimulation with immobilized anti-CD3 and anti-CD28 mAbs (Figure 1b). Stimulation of Th1 cells induced high CD70 expression levels that peaked at 48–72 hours and then gradually decreased. In contrast, no significant expression of CD70 was induced on Th2 cells by the same stimulation (Figure 1c). Similarly, although CD70 expression was not observed on resting or activated D10G4 cells, CD70 was expressed on activated 28-4 cells (Figure 1d). Furthermore, both resting and activated human Th1 cell line HK-12 cells expressed abundant CD70 (Supplementary Figure S1 online).

### Engagement of CD27 increases the proliferation and cytokine production of naive $CD4^+$ T cells, but not of Th1 or Th2 cells

To examine the costimulatory function of CD27, naive $CD4^+$ T cells, Th1 and Th2 cell lines were stimulated with immobilized anti-CD3 mAb in the presence of coimmobilized anti-CD27 mAb or control IgG. Engagement of CD27 by anti-CD27 mAb significantly increased the proliferation and the IFN-γ and IL-4 production of naive $CD4^+$ T cells (Figure 2a), whereas it did not significantly affect the proliferation or cytokine production of Th1 or Th2 cells (Figure 2b). To further examine whether CD27/CD70 signaling is involved in T–T cell interactions, naive $CD4^+$ T cells or Th1 cells were stimulated with immobilized anti-CD3 and anti-CD28 mAb or with immobilized anti-CD3 mAb

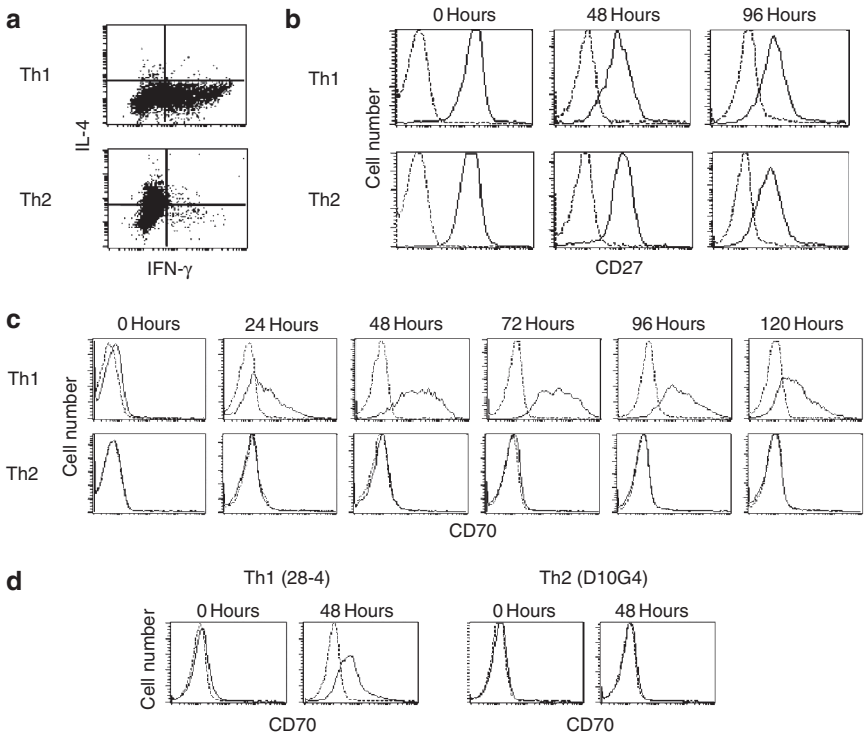


**Figure 1. Expression of CD27 and CD70 on Th1 and Th2 cells.** (**a**) Cytoplasmic staining for IFN-γ and IL-4. Th1 and Th2 cell lines were stimulated with PMA plus ionomycin for 24 hours. (**b**, **c**) Th1 and Th2 cell lines were stimulated with anti-CD3 and anti-CD28 mAbs for the indicated periods. Cells were stained with (**b**) anti-CD27 mAb or with (**c**) anti-CD70 mAb (solid lines). (**d**) Murine Th1 (28-4) and Th2 (D10G4) clones were stimulated with anti-CD3 and anti-CD28 mAbs for 48 hours, and then stained with anti-mouse or human CD70 mAb (solid lines). (**b**–**d**) Broken lines indicate background staining with control antibodies. All data are representative of three independent experiments with similar results. PMA, phorbol 12-myristate 13-acetate; Th, T-helper.

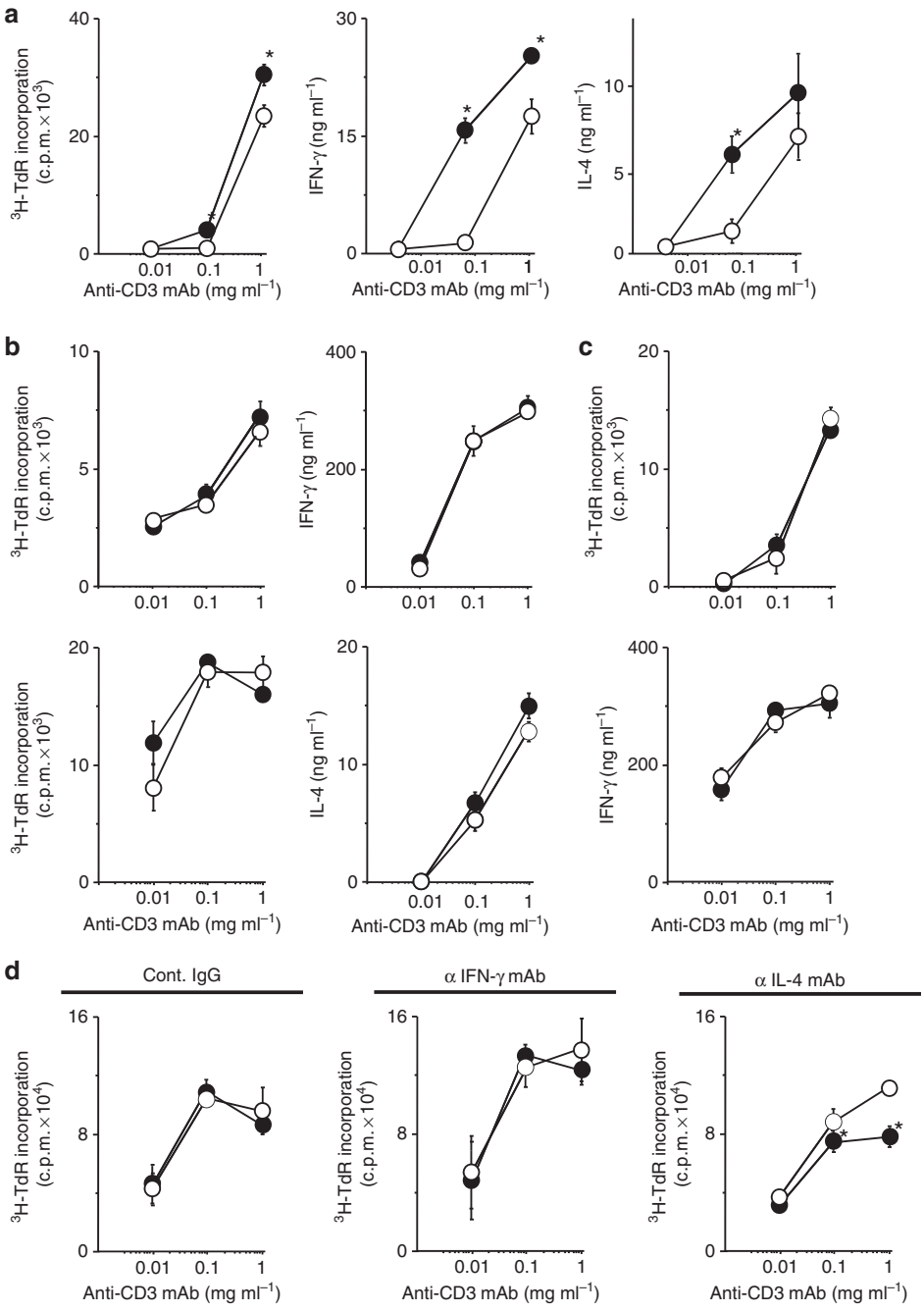


**Figure 2. Engagement of CD27 increases the proliferation and cytokine production of naive CD4$^+$ T cells, but not of Th1 or Th2 cells.** (**a**) Naive CD4$^+$ T cells, (**b**, upper panels) Th1, or (**b**, lower panels) Th2 cell lines were stimulated with anti-CD3 mAb in the presence of anti-CD27 mAb (●) or control IgG (○). (**c**) Th1 cell lines were stimulated with anti-CD3 mAb in the presence of anti-CD70 mAb (●) or control IgG (○). (**d**), Naive CD4$^+$ T cells were stimulated with anti-CD3 and anti-CD28 mAbs in the presence of the indicated mAbs and anti-CD70 mAb (●) or control IgG (○). Cells were assessed for proliferation and production of IFN-γ and/or IL-4. Similar results were obtained in three independent experiments. *$P$<0.05 compared with the control IgG group. Cont., control; Th, T-helper.

alone, respectively, in the presence of soluble anti-CD70 mAb or control IgG. Although we confirmed that CD70 was induced on naive CD4$^+$ T cells and Th1 cells by those stimulations (data not shown), no significant differences between control IgG and anti-CD70 mAb were observed in terms of naive CD4$^+$ T-cell proliferation (Figure 2d) or Th1-cell proliferation and IFN-γ production (Figure 2c). However, in the presence of anti-IL-4 mAb, anti-CD70 mAb significantly decreased naive CD4$^+$ T-cell expansion (Figure 2d), suggesting that IL-4 produced by activated T cells restricts T–T cell interaction by CD70/CD27 signaling.

### CD70 is important for OVA-specific T-cell responses

To clarify the role of CD27/CD70 in CD4$^+$ T-cell responses *in vivo*, we used a DTH model (Kearney *et al.*, 1994).

DO11.10 (KJ1-26$^+$) CD4$^+$ T cells were adoptively transferred into BALB/c mice, and 2 days later, OVA/complete Freund's adjuvant (CFA) was injected subcutaneously (Kearney *et al.*, 1994). Intraperitoneal (i.p.) administration of anti-CD70 mAb significantly decreased the number of Ag-specific (KJ1-26$^+$) CD4$^+$ draining lymph node (DLN) T cells at day 6 compared with control Ig-treated mice (Figure 3a). When DO11.10 CD4$^+$ T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE) before adoptive transfer, immunization with OVA/CFA dramatically induced cell division of KJ1-26$^+$ T cells, which was manifested by reduced CFSE levels (Figure 3b). Treatment with anti-CD70 mAb did not affect the cell division in KJ1-26$^+$ T cells (Figure 3b), whereas it significantly increased the percentage of apoptotic (annexin V$^+$) cells in KJ1-26$^+$ T cells as compared with control IgG-treated mice (Figure 3c). To explore the functional contribution of CD70 to cytokine production by KJ1-26$^+$ T cells, DLN cells were restimulated with the $OVA_{323–339}$ peptide *in vitro*. Strikingly, both the frequency of IFN-γ-secreting cells and the level of IFN-γ production were significantly reduced in cultures derived from anti-CD70 mAb-treated mice as compared with control IgG-treated mice (Figure 3d). These results suggest that the CD27/CD70 interaction has an important role in supporting Ag-specific naive CD4$^+$ T-cell expansion by allowing the primed CD4$^+$ T cells to survive successive divisions *in vivo*.

We next examined CD70 expression in this model. The number of CD70$^+$ KJ1-26$^+$ T cells and CD70 mRNA expression levels in CD4$^+$ T cells were dramatically increased after primary and secondary immunization with OVA/CFA (Figure 4a and b). Notably, CD70$^+$ cells were only detected in the CFSE$^{low}$ KJ1-26$^+$ T-cell fraction, indicating that cell surface CD70 was expressed on activated T cells undergoing cell divisions. Interestingly, CD70 expression was observed on CD4$^+$ T cells 3 days after the first immunization, whereas its expression on CD11c$^+$ DCs peaked on day 1 (Figure 4c).

To confirm whether CD70 expressed on T cells is functional, CD4$^+$ T cells were purified from DLN cells harvested 3 days after immunization, and cultured in the presence of soluble anti-CD70 mAb or control IgG for 3 days. As expected, anti-CD70 mAb significantly decreased CD4$^+$ T-cell expansion (Figure 4d), suggesting that CD70 expressed on CD4$^+$ T cells is functional in T–T cell interaction.

**CD70 is important for the induction of Th1-predominant CHS**

To explore the role of CD27/CD70 in Th1-mediated diseases *in vivo*, we investigated DNFB-induced CHS. It has been

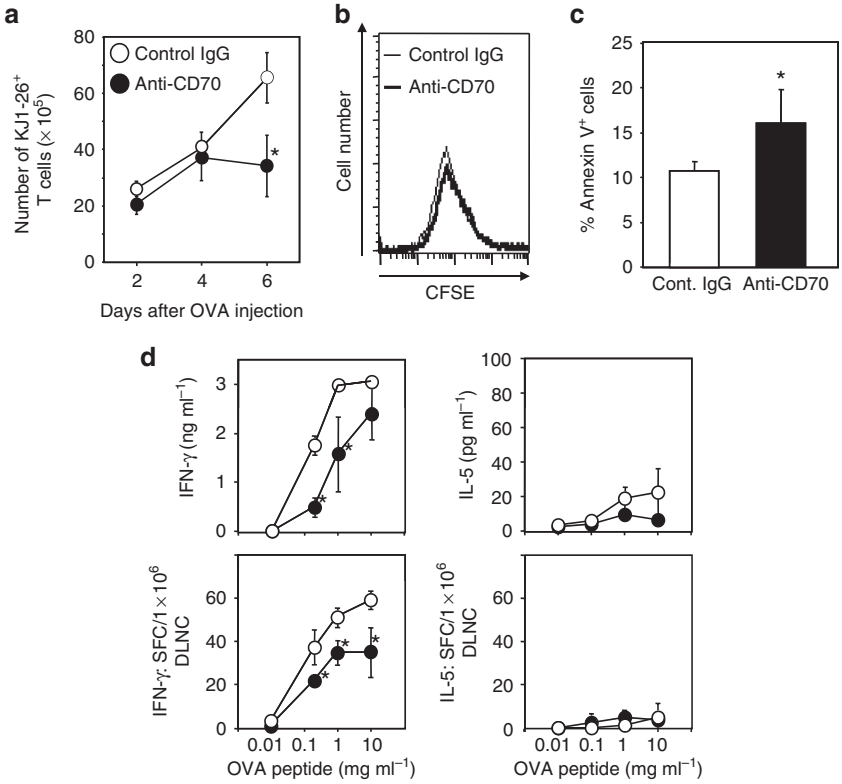


**Figure 3. CD70 is important for naive T-cell survival in Th1 immune response.** BALB/c recipients of DO11.10 T cells were immunized with OVA/CFA and administered with control IgG (○) or anti-CD70 mAb (●). DLNs were harvested at (**a**) the indicated time points or (**b–e**) 6 days after immunization. (**a**) The total number of KJ1-26$^+$ CD4$^+$ T cells in DLNs. (**b**) DO11.10 T cells were labeled with CFSE before adoptive transfer. Cell division of KJ1-26$^+$ CD4$^+$ T cells was compared by CFSE dilution. (**c**) Incidence of apoptosis in KJ1-26$^+$ DLN cells as determined by annexin V binding. (**d**) DLN cells were restimulated with the $OVA_{323–339}$ peptide and assessed for cytokine production by ELISA (upper panels) and ELISPOT (lower panels). Similar results were obtained in three independent experiments. *$P<0.05$ compared with the control IgG group. Cont., control; CFSE, carboxyfluorescein diacetate succinimidyl ester; DLN, draining lymph node; OVA, ovalbumin; SFC, spot-forming cells; Th, T-helper.

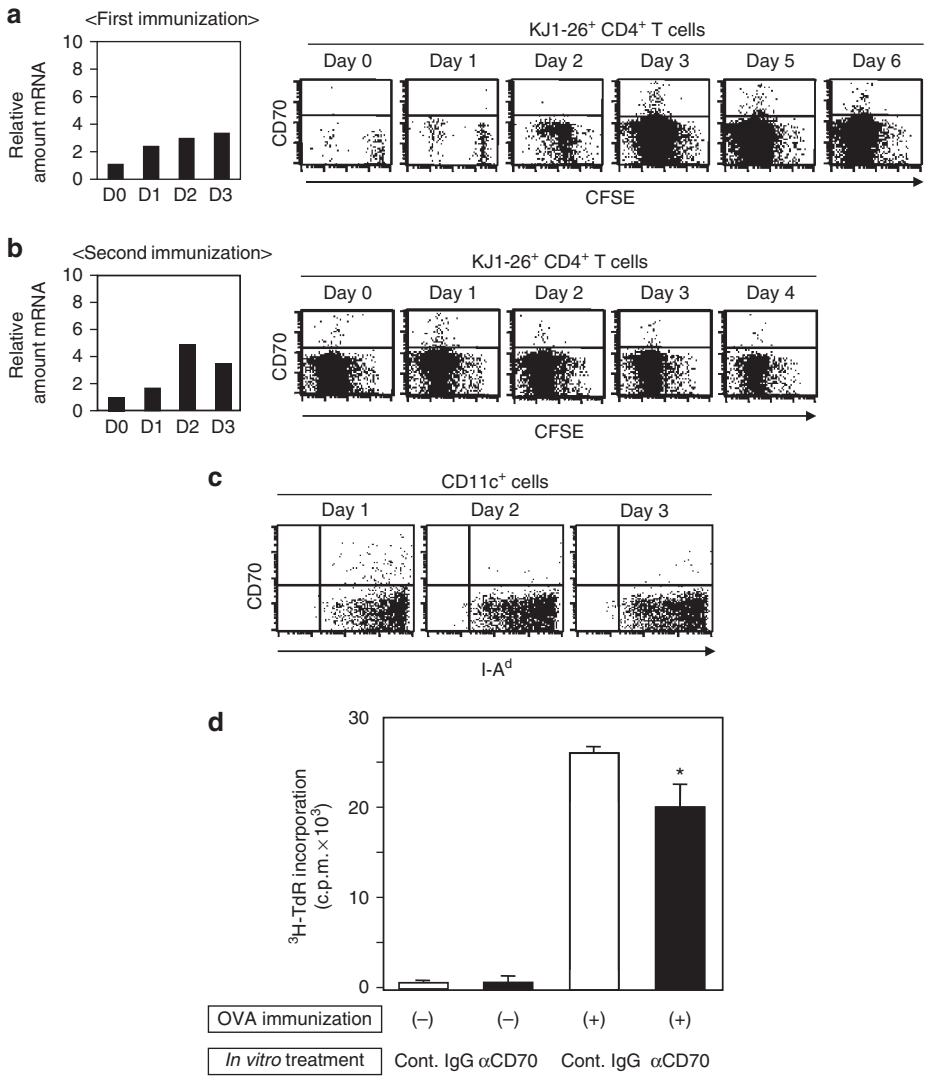


**Figure 4. CD70 expression *in vivo*.** BALB/c recipients of DO11.10 $CD4^+$ T cells were immunized with OVA/CFA (**a**, **c**, **d**) or re-immunized 7 days after the first immunization (**b**), and DLN cells were harvested at the indicated time points. (**a** and **b**, upper panels) Real-time PCR quantification of CD70 mRNA. For purified $CD4^+$ T cells isolated from DLNs, CD70 mRNA copy numbers were normalized to G3PDH mRNA. Results are presented as fold induction over baseline values obtained at day 0. (**a** and **b** lower panels) DO11.10 $CD4^+$ T cells were labeled with CFSE before adoptive transfer. Data show CD70 expression on electronically gated $CD4^+$ KJ1-$26^+$ DLN cells. (**c**) DLN cells were harvested at the indicated time points. Data show CD70 expression on electronically gated $CD11c^+$ cells. (**d**) $CD4^+$ T cells were purified from DLN cells harvested 3 days after immunization. Cells were cultured in the presence of anti-CD70 mAb (black bar) or control IgG (white bar) for 3 days, and assessed for proliferation. Similar results were obtained in three independent experiments. CFSE, carboxyfluorescein diacetate succinimidyl ester; Cont., control; DLN, draining lymph node; OVA, ovalbumin.

shown using CD8−/− and CD4−/− mice that both $CD4^+$ Th1 and $CD8^+$ T cells are crucial effector cells in CHS responses to DNFB (Wang *et al.*, 2000). Interestingly, treatment with anti-CD70 mAb at sensitization significantly inhibited ear swelling as compared with control Ig-treated mice (Figure 5a), whereas treatment at challenge did not significantly affect ear swelling (Figure 5b). DLN cells isolated 5 days after DNFB sensitization produced a high level of IFN-γ and a low level of IL-4 in response to DNBS (2,4-dinitrobenzene-sulfonic acid, a water-soluble analog of DNFB) restimulation (Figure 5c). The frequency of IFN-γ-secreting cells and the level of IFN-γ production were significantly reduced in cultures derived from mice treated with anti-CD70 mAb as than in those from control IgG-treated mice (Figure 5c and d). In contrast, the *in vitro* addition of anti-CD70 mAb during DNBS restimulation did not significantly affect the frequency of IFN-γ-producing cells recovered from DNFB-sensitized mice (Figure 5d). A small population of $CD4^+$ T cells and $CD8^+$ T cells isolated from DLNs expressed CD70 (Figure 5e), and CD70 mRNA was also induced in $CD11c^+$ DCs in DLNs after sensitization (Figure 5f). Notably, we could not detect significant CD70 expression on Th2, Th17, or regulatory T cells isolated from DLNs (Supplementary Figure S2 online). These findings

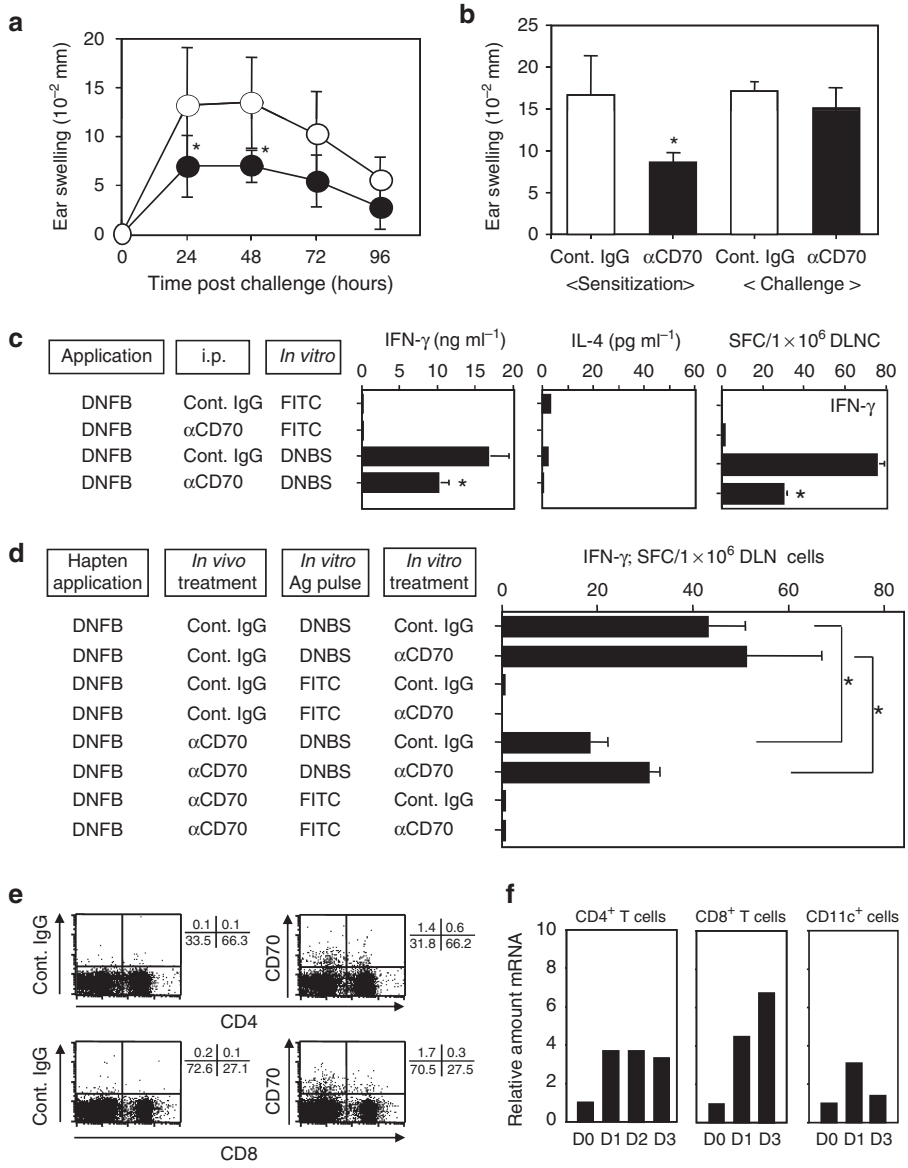


**Figure 5. Administration of anti-CD70 mAb at sensitization inhibits CHS.** CHS against DNFB was induced. Control IgG or anti-CD70 mAb was injected intraperitoneally (**a**–**d**) 2 hours before and 2 days after sensitization or (**b**) 2 hours before challenge. Ear thickness was evaluated at the (**a**) indicated time points or (**b**) 24 hours after challenge. (**c** and **d**) DNBS- or FITC-pulsed DLN cells from each group of mice were cultured and assessed for cytokine production. (**d**) Cells were cultured in the presence of anti-CD70 mAb or control IgG. **(e)** Data show CD70 expression on electronically gated CD3$^+$ DLN cells. (**f**) Real-time PCR quantification of CD70 mRNA for indicated cells purified from DLNs. Results are presented as fold induction over baseline values of each cell type obtained at day 0. *$P<0.05$ compared with the control IgG group. Data are representative of several experiments with similar results. CHS, contact hypersensitivity; Cont., control; DLN, draining lymph node; i.p., intraperitoneal; SFC, spot-forming cells.

suggested that CD27/CD70 signaling may support Ag-specific primed T-cell expansion during the sensitization phase of the CHS response.

### Blockade of the CD70/CD27 interaction did not affect Th2-mediated asthmatic responses

To clarify the role of CD27/CD70 in Th2-type immune responses *in vivo*, we used a murine model of asthma caused by allergen-specific Th2 cells (Wills-Karp, 1999). As shown in Figure 6a–c, administration of anti-CD70 mAb did not significantly affect the serum anti-OVA IgE and IgG1 levels, Th2 cytokine levels in bronchoalveolar lavage fluid, and inflammatory cell infiltration around the bronchioles, as compared with those in control IgG-treated mice. In addition, when splenic T cells were restimulated with OVA *in vitro*, the production of IFN-γ, IL-4, and IL-5 was not significantly inhibited in anti-CD70 mAb-treated mice (Figure 6d). These results indicate that unlike Th1 responses, the blockade of CD70 during the induction phase did not affect the development of asthmatic Th2 responses. Consistent with these findings, we could not detect significant CD70 expression on CD4$^+$ cells obtained from DLNs and splenic cells after last immunization with OVA in asthmatic responses (Figure 6e and f).

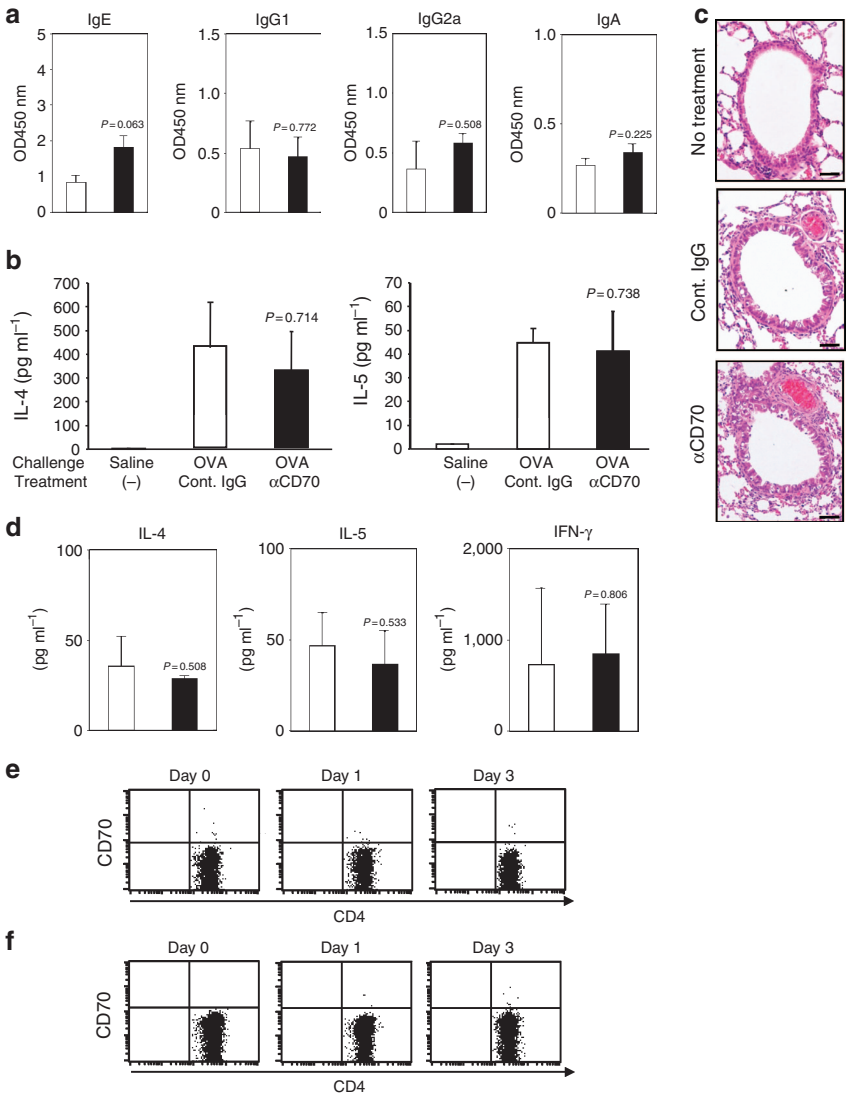


**Figure 6. Administration of anti-CD70 mAb at sensitization does not affect asthmatic responses.** BALB/c mice were immunized with OVA/alum on days 0 and 12, and then challenged with saline or OVA on days 22, 23, and 24. Control IgG or anti-CD70 mAb was injected intraperitoneally every 3 days from day 0 to day 18. (**a**) Twenty-four hours after the last OVA challenge, OVA-specific IgE, IgG1, IgG2a, and IgA serum antibody levels were determined by ELISA. (**b**) Cytokine levels in the BALF were measured by ELISA. (**c**) Histological examination of the lungs by H–E staining. Scale bar = 2 μm. (**d**) Splenocytes from sensitized mice treated with either control IgG or anti-CD70 mAb were stimulated with OVA *in vitro*. Cytokine levels in the supernatants were measured by ELISA. *P*-value: compared with the control IgG group. (**e**, **f**) DLN (**e**) and splenic (**f**) cells obtained 0, 1, and 3 days after the last immunization were stained with anti-CD70 and anti-CD4 mAb. Data showing the staining of electronically gated $CD4^+$ T cells. Data are representative of several experiments with similar results. alum, aluminum hydroxide; BALF, bronchoalveolar lavage fluid; Cont., control; DLN, draining lymph node; H–E, hematoxylin and eosin; OVA, ovalbumin.

## DISCUSSION

We demonstrated that *in vivo* blockade of CD70/CD27 signaling significantly inhibited antigen-specific naive $CD4^+$ T-cell expansion and Th1-cell generation, and increased the number of apoptotic cells in Ag-specific T cells without affecting cell division (Figure 3). However, we found that CD27/CD70 signaling was not critically involved in either [$^3$H]-thymidine incorporation or cytokine production by Th1 or Th2 cells (Figure 2). These findings suggest that CD27 signaling may be required for survival of primed T cells, but not of polarized Th1 or Th2 cells, and have a critical role in supporting the survival of Ag-primed T cells during the induction phase of Th1-type immune responses. This notion is supported by the fact that inhibitory effects of anti-CD70 mAb were only observed during the sensitization, but not the challenge, phase of the Th1- and Tc1-mediated CHS response.

We found that during the DTH response, CD70 expression on $CD4^+$ T cells was observed 3–6 days after the first immunization, whereas CD70 expression on DCs peaked at 1 day after immunization (Figure 4). Similarly, during the CHS response, abundant CD70 mRNA expression was observed in T cells at 3 days after sensitization, whereas its expression rapidly decreased in $CD11c^+$ DCs/Langerhans cells at day 3 (Figure 5). As it has been reported for both

responses that Ag-bearing DCs and Langerhans cells rapidly disappear from the DLN 48–72 hours after immunization (Ingulli *et al.*, 1997; Kawamura *et al.*, 1999), it is possible that the rapid loss of CD70 on DCs/Langerhans cells, probably induced by interaction with CD40L-expressing $CD4^+$ T cells (Taraban *et al.*, 2004; French *et al.*, 2007), may be due to the disappearance of these cells from the DLN. In humans, a direct cellular communication between $CD45RO^+$ and $CD45RA^+$ T cells through CD27/CD70 has been proposed (Agematsu *et al.*, 1995). It is possible that CD70 expressed on Th1 cells contributes to an amplification loop of polarized Th1-type immune responses through Th1 cell-naive T-cell interactions. Indeed, we found that in the absence of IL-4, blockade of CD27/CD70 signaling through T–T cell interaction significantly decreased naive $CD4^+$ T-cell expansion (Figure 2d). Taken together, the above findings suggest that the CD27 function is controlled by the differentially regulated kinetics of CD70 expression on DCs and activated $CD4^+$ T cells during the priming phase of Th1 immune responses *in vivo*. It has been demonstrated that administration of the recombinant soluble CD70 protein during antigen stimulation resulted in a massive expansion of Ag-specific $CD8^+$ T cells *in vivo* (Rowley and Al-Shamkhani, 2004). Moreover, T cell-specific CD70 transgenic mice infected with the influenza virus showed enhanced numbers of effector memory $CD4^+$ T cells in B-cell follicles (Beishuizen *et al.*, 2009), thus suggesting that like studies in humans (Agematsu *et al.*, 1995), CD70 expressed on T cells is functional and provides CD27 signaling in activating $CD4^+$ T cells *in vivo*. Nevertheless, these mice in steady state did not show enhanced effector memory formation of $CD4^+$ T cells, despite the fact that the expression of Ki-67 was upregulated in effector memory $CD4^+$ T cells (van Gisbergen *et al.*, 2009). Although we demonstrated that CD70 expressed on $CD4^+$ T cells is functional in T–T cell interaction (Figures 2d and 4d), further studies are required to determine the functional significance of CD70 on $CD4^+$ T cells *in vivo*.

In this study, we have shown that blockade of the CD70/CD27 interaction inhibits Th1-mediated DTH and CHS responses, but does not affect Th2-mediated asthmatic responses. We also found that CD70 is expressed on Th1, but not on Th2, cells and clones, and that IL-4 strongly suppressed induction of CD70 expression on $CD4^+$ T cells (Figure 1 and data not shown). In addition, we could detect *ex vivo* CD70 expression on $CD4^+$ cells in Th1-mediated DTH and CHS, but not in Th2-mediated asthmatic responses (Figures 4, 5 and 6). In this regard, it has been demonstrated that induction of CD70 expression on human monocyte-derived DCs by lipopolysaccharide required an absence of IL-4 (Iwamoto *et al.*, 2005). On the other hand, CD70 on splenic DEC-$205^+$ DCs is important for the induction of IL-12-independent IFN-γ production by $CD4^+$ T cells *in vivo* (Soares *et al.*, 2007), and in the presence of IL-12, CD27 signaling promotes Th1 development *in vitro* (van Oosterwijk *et al.*, 2007). Nevertheless, human CD70 expressed on DCs promotes the development of $CD4^+$ T cells that produce both Th1 and Th2 cytokines (Hashimoto-Okada *et al.*, 2009). One possible explanation for the above findings is that IL-4 produced by $CD4^+$ T cells critically restricts CD70 expression on both DCs and $CD4^+$ T cells in type 2 immune responses *in vivo*, and therefore only in the setting of IL-4 paucity (e.g., Th1-type immune responses), CD70/CD27-mediated DC-T cell or T–T cell interactions have a role in activating naive $CD4^+$ T cells. This idea may, at least in part, account for the discordant results in previous reports regarding *in vivo* roles of the CD70/CD27 interaction in various $CD4^+$ T cell-mediated disease models, including inflammatory bowel disease (Sumi *et al.*, 2008) and experimental allergic conjunctivitis (Manocha *et al.*, 2009) models.

## MATERIALS AND METHODS

### Animals

Six-week-old female BALB/c and DO11.10 mice were purchased from Charles River Japan (Atsugi, Japan) and the Jackson Laboratory (Bar Harbor, ME), respectively. All mice were used in accordance with the guidelines of the Committee on Animals of Juntendo University and the University of Yamanashi.

### Reagents and antibodies

Ovalbumin, lipopolysaccharide, PMA, ionomycin, and CFA were purchased from Sigma (St Louis, MO). The cell-permeant fluorescent dye CFSE was purchased from Molecular Probes (Eugene, OR). An anti-murine CD70 mAb (FR70, rat IgG2b) was prepared as described previously (Oshima *et al.*, 1998). The other mAbs were purchased from BD Pharmingen (San Jose, CA).

### Cell lines and tissue culture

Th1 and Th2 cell lines were originated from naive $CD4^+$ T cells prepared from the spleen or lymph node of OVA-specific TCR-αβ transgenic mice (DO11.10) as described previously (Watanabe *et al.*, 1997). T-cell clones used were: HK-12 (human Th1 clone derived from phytohemagglutinin-stimulated peripheral blood lymphocyte), 28-4 (murine Th1 clone specific for KLH), and D10G4 (murine Th2 clone, specific for conalbumin). T-cell lines and clones were maintained in RPMI 1640 medium containing 10% fetal calf serum, 10 mM HEPES, 0.25 $\mu g\,ml^{-1}$ gentamycine, 2 mM L-glutamine (complete medium), and 50 Units $ml^{-1}$ recombinant human IL-2 (Shionogi, Osaka, Japan). Naive T cells, T-cell lines, and clones were stimulated with immobilized anti-CD3 mAb (0.01–10 $\mu g\,ml^{-1}$) in the presence or absence of immobilized control IgG or mAbs (10 $\mu g\,ml^{-1}$) against CD28 and CD27 or with PMA (50 $ng\,ml^{-1}$) plus ionomycin (500 $ng\,ml^{-1}$).

### OVA-specific DTH responses and mAb treatment *in vivo*

DO11.10 T cells were identified by staining with KJ1-26 mAb (Caltag, Burlingame, CA) and prepared for adoptive transfer as described previously (Kearney *et al.*, 1994). In brief, transgenic lymph nodes and spleen cells depleted of $CD8^+$ T cells were adoptively transferred by intravenous injection into BALB/c mice such that $2 \times 10^6$ KJ1-$26^+$ T cells were given to each recipient. Mice were injected subcutaneously with 100 μg OVA emulsified in CFA (upper and lower back) 2 days later. Groups of five mice received i.p. injection of 250 μg per mouse anti-CD70 mAb or control IgG 2 hours before and 48 hours after immunization. In some experiments, DO11.10 T cells were stained with 2.5 μM CFSE before adoptive transfer. To determine cytokine production, $4 \times 10^6$ DLN

cells collected 6 days after immunization were cultured in the presence of 0–10 μg ml$^{-1}$ $OVA_{323–339}$ peptide for 48 hours.

### Contact hypersensitivity

A volume of 20 μl of 0.5% DNFB dissolved in acetone:olive oil (4:1) was painted on the shaved abdomen of mice at days 0 and 1, and then the ears of mice were challenged with 10 μl of 0.2% DNFB on day 5. Ear thickness was measured as described previously (Nuriya *et al.*, 2001). Groups of five mice received i.p. injection of 250 μg per mouse anti-CD70 mAb or control IgG 2 hours before and 48 hours after sensitization or 2 hours before challenge. To determine cytokine production, DLN cells collected from mice 5 days after sensitization or challenge were incubated with 10 mM DNBS (Tokyo Kasei, Tokyo, Japan) or 0.3 mg ml$^{-1}$ FITC (isomer-I, Dojindo, Kumamoto, Japan) for 10 minutes as described previously (Nuriya *et al.*, 2001).

### Induction of allergic airway inflammation and mAb treatment *in vivo*

Groups of five mice were sensitized by i.p. injection of 50 μg OVA with 1 mg aluminum hydroxide (alum) on days 0 and 12. On days 22, 23, and 24, mice received intranasal challenges of either 20 μl of saline or 20 μl of saline containing 100 μg of OVA. Twenty-four hours after the last OVA challenge, the trachea was cannulated with a polyethylene tube through which the lungs were gently lavaged with 0.8 ml phosphate-buffered saline containing 0.1% BSA three times (2.4 ml total of bronchoalveolar lavage fluid). On day 25, OVA-specific IgE, IgG1, IgG2a, and IgA serum antibody levels were determined by ELISA (Hoshino *et al.*, 2003). The lungs were removed from mice 24 hours after the last OVA challenge, fixed in 10% neutral-buffered formalin, and embedded in paraffin. Sections of 5-μm thickness were stained with hematoxylin and eosin. To determine cytokine production, splenocytes collected on day 25 were incubated with OVA (100 μg ml$^{-1}$) for 72 hours. To examine the effect of anti-CD70 mAb, mice were i.p. injected with 300 μg of anti-CD70 mAb or control IgG every 3 days from day 0 to day 18.

### Real-time PCR

$CD4^+$ T cells, $CD8^+$ T cells, and DCs were positively selected from DLN cells using anti-CD4, anti-CD8, and anti-CD11c magnetic beads, respectively, and MACS columns (Militenyi Biotec, San Diego, CA). Relative CD70 mRNA expression was determined by real-time PCR using an ABI PRISM 5500 Sequence Detection System (Applied Biosystems, Framingham, MA) with SYBR Green I dye (Qiagen, Tokyo, Japan). Total RNA was isolated using TRIzol (Invitrogen Life Technologies, Carlsbad, CA), and cDNA was synthesized using the SuperScript system (Invitrogen Life Technologies). Primers corresponding to mouse CD70 and G3PDH were designed by Takara Bio (Shiga, Japan) as follows: 5′-AGCGGACTACTCAGTAAGCAGCAAC-3′ and 5′-CAGCTCTGGTCCGTGTGTGAA-3′ for CD70, and 5′-AAATGGTGAAGGTCGGTGTG-3′ and 5′-TGAAGGGGTCGTTGATGG-3′ for G3PDH. Cycle threshold numbers (Ct) were derived from the exponential phase of PCR amplification. Fold differences in the expression of gene $x$ in the cell populations $y$ and $z$ were derived by $2^k$, where $k = (Ctx - Ct_{G3PDH})y - (Ctx - Ct_{G3PDH})z$.

### Flow cytometry

Cells ($5 \times 10^5$) were stained with the indicated mAbs (10 μg ml$^{-1}$) for 30 minutes at 4°C and analyzed on a FACSCalibur (Becton Dickinson, San Jose, CA). FITC-labeled annexin V (R&D Systems, Minneapolis, MN) was used for detection of apoptosis. To assess intracellular cytokine production, cells were surface stained, fixed and permeabilized, and then stained intracellularly for IFN-γ, IL-4, and IL-17.

### *In vitro* proliferation and cytokine assays

Naive CD4 T cells, Th1, and Th2 cells stimulated as described above were cultured for 72–96 hours and the proliferative response was measured by [$^3$H]-thymidine ($^3$H-TdR) uptake (0.5 μCi per well) for the last 16 hours. Cell-free culture supernatants and cells were assayed using cytokine-specific ELISA and ELISPOT assay kits, respectively (R&D Systems).

### Statistical analyses

Significant differences between experimental groups were analyzed by Student's *t*-test. *P*-values $<0.05$ were considered significant.

## CONFLICT OF INTEREST

The authors state no conflict of interest.

## ACKNOWLEDGMENTS

We thank Naotaka Shibagaki and Hiroyuki Matsue for helpful discussions, and Izumi Ishikawa for technical assistance.

## SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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